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CORRELATION OF PHOSPHOINOSITIDE HYDROLYSIS WITH EXFLAGELLATION IN THE MALARIA MICROGAMETOCYTE

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ABSTRACT: Cellular responses to growth factors, hormones, and other agonists have been shown in many animal cell systems to be mediated by the signal transduction cascade controlled by phospholipase C. One such response, calcium mobilization, is regulated by the concerted effect of several specific inositol (poly)phosphates. Another response, protein phosphorylation, is regulated by other phospholipase C (PLC) hydrolysis products. Mature gametocytes are specialized cells primed for transformation into gametes immediately upon removal from the vertebrate bloodstream, thereby initiating the sexual cycle in a vector mosquito. This study showed that PLC hydrolysis products, inositol (1,4,5)triphosphate and diacylglycerol, are correlated with the initial events of flagellar development; they are implicated in synchronizing this crucial transformation for the parasite and hence the continued transmission of the parasite, which leads to this debilitating disease.

Plasmodium falciparum gametocytes do not emerge from erythrocytes within the bloodstream of a vertebrate host, but they rapidly transform into gametes soon after ingestion by a vector mosquito, and then they combine to form a zygote, initiating the sexual cycle. Emerged macrogametocytes transform to a single macrogamete, whereas the microgametocytes undergo a striking phenomenon called exflagellation in which as many as 8 microgametes are produced and released from a single mature microgametocyte. Although this landmark discovery gave malaria its scientific basis, it is the asexual rather than the sexual cycle that has generated most of the subsequent research in this field. The reason for this skewed interest may be attributed to the fact that the morbidity and mortality associated with plasmodial infections are due solely to the asexual cycle, as circulating gametocytes are not known to cause any symptoms. However, it is the sexual cycle with its precise timing of gametogenesis within the midgut of a potential vector that makes transmission of the parasite possible. Moreover, this cycle is equally vital from an evolutionary standpoint in that it provides for the possibility of gene recombination (Walliker et al., 1987).

Some specialized mammalian cells such as platelets and mast cells respond in a predetermined way to specific stimuli. Thus, a responsive cell living in a complex environment with diverse stimuli will respond in an appropriate manner to a specific stimulus. After reception of the extracellular signal, a series of reactions is set in place that ultimately activates the internal machinery of the cell to perform the biologic function. In this manner, an external stimulus is linked to a specific cellular response, i.e., stimulus response coupling (Berridge, 1987; Putney and Hughes, 1989). The implication of phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate in cell membrane signal transduction systems paved the way for the current understanding of the biochemical reactions that underlie this process (Hokin and Hokin, 1953). Phosphoinositide hydrolysis has now been shown to be involved in the timely induction of several biological functions, e.g., activation of platelets (Lapetina, 1988), mast cells, and leukocytes (Sadler and Badwey, 1988) and secretion of hormones (Catt and Balla, 1989), but there is no published report of such reactions occurring in protozoa. However, protozoan parasites are known to engage in intricately coordinated life cycles during which timed cellular transformations take place in specified environments (Trager, 1986). Hence, discriminatory signals must exist in these environments along with biochemical mechanisms for coupling these signals to appropriate parasite transformations. The mature *P. falciparum* gametocyte, in particular, is a specialized cell primed for transformation into gametes immediately after release from the vertebrate host and ingestion by the mosquito or exposure to ambient air. As in responsive mammalian cells, phosphoinositide hydrolysis products may be involved in synchronizing this crucial parasite transition. This hypothesis was tested

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by monitoring the kinetics of the formation of these hydrolysis products in stimulated microgametocytes from cultures of the human malarial parasite.

MATERIALS AND METHODS

The NF54 strain of *P. falciparum* (Delemarre-Van de Waal and de Waal. 1981) was cultured using the method of Trager and Jensen (1976) as modified by Ifediba and Vanderberg (1981). Gametocytes first appear by day 3 or 4 and reach maturity and the capability to exflagellate (stage V of Hawking et al. [1971]) between days 9 and 14. Parasitemias. both asexual and gametocytes, normally ranged between 3 and 8%. Between 1 and 2 × 10⁰ very early stage V gametocytes were isolated on a discontinuous Percoll gradient (Knight and Sinden. 1982). Less than 3% of the parasites from the gradient were asexuals. After washing 3 × in the medium. the gametocytes were placed in 3. T-75 culture flasks with 15 ml of medium (RPMI-1640. supplemented with 25 mM HEPES. 50 μg ml hypoxanthine. 0.2% sodium bicarbonate. and 10% human serum) and 100 μCi of [¹H]myoinositol per flask.

After 1–3 days (depending on the developmental stage of the gametocytes). the gametocytes were removed, concentrated by centrifugation. and supernatant solution was removed. and 5 ml suspended animation medium lacking bicarbonate (Carter and Nijout. 1977) containing 10 mM LiCl was immediately mixed with the pellet. The gametocytes were centrifuged again and activated with complete medium containing 25 mM NaHCO₃. 1 μM CaCl₂. and 10 mM LiCl, at pH 8.0. One-milliliter samples were taken at various times in the process of exflagellation (see legend to Fig. 1) from 30 sec through 35 min: the reaction was stopped at these times by addition of 100 μl of perchloric acid. and samples were frozen on dry ice (Downes et al.. 1986). The perchloric acid was removed using freon octylamine (Downes et al.. 1986) and the inositol (poly)phosphates were separated by high-performance liquid chromatography (HPLC) using a Whatman SAX-PartiSphere column with elution buffers of water to 1.4 M ammonium phosphate essentially by the procedure of Dean and Moyer (1988). Calibration of the column was achieved using inositol (poly)phosphate standards that were purchased from E. I. Dupont & Nemours and Co.. Inc. (Boston. Massachusetts).
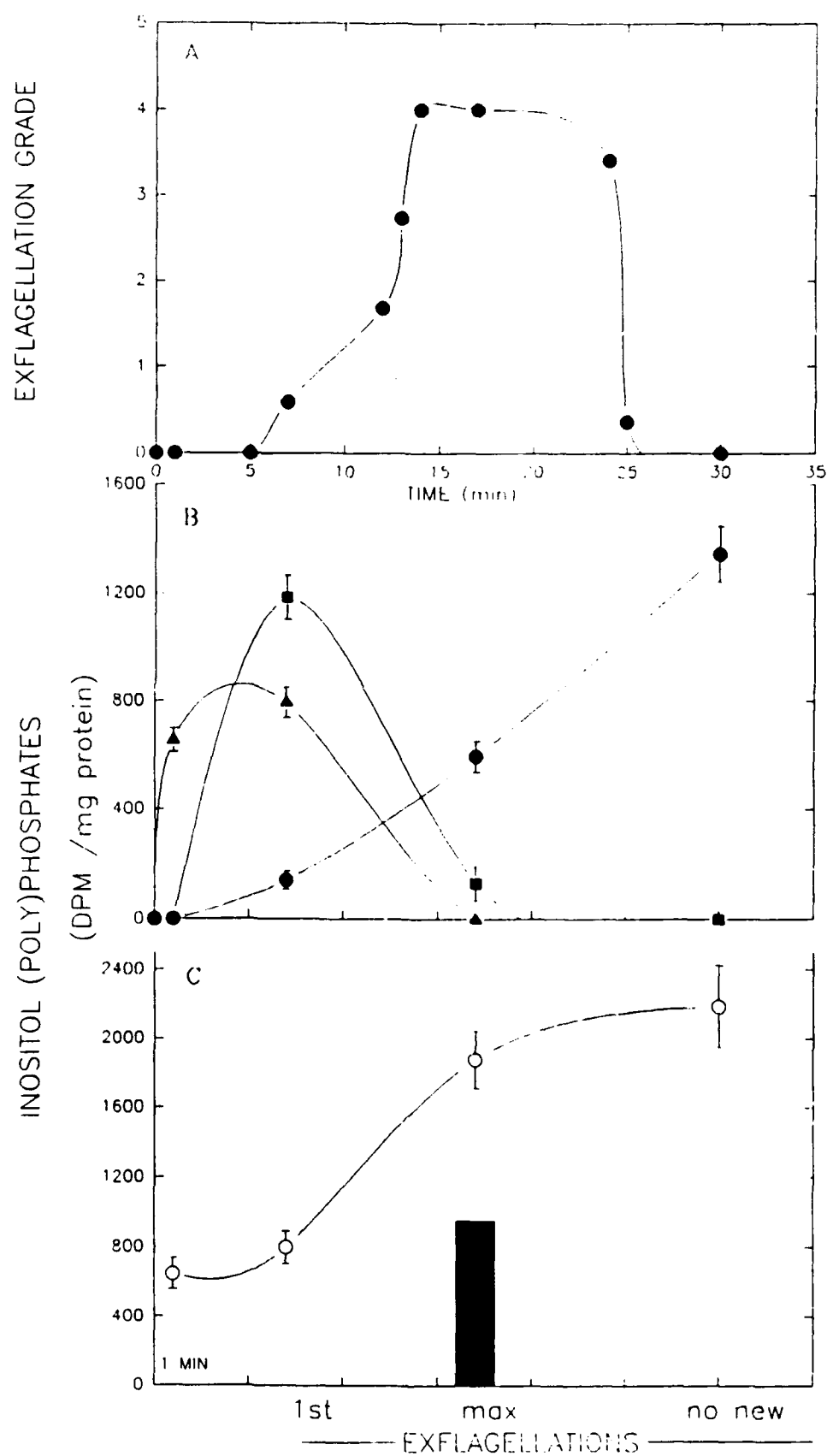
The diacylglycerol studies were performed similar to the method described above except 100 μCi [¹H]glycerol were added per flask in place of [¹H]myoinositol. After 1–3 days. the same procedure was followed. In these experiments the reaction was stopped by the addition of CHCl₃. MeOH (2 1) containing 0.1 M hydrochloric acid (3 times the reaction volume) and a Bligh–Dyer extraction (Bligh and Dyer. 1959) performed. The samples were dried and dissolved in 50 μl CHCl₃. Ten microliters was spotted on channeled silica thin-layer chromatography (TLC) plates and run using a solvent designed to separate mono- and diacylglycerols and fatty acids (Chudzik and Stanacev. 1983). It consisted of a solution of toluene ethyl ether ethanol/ammonium hydroxide (54.2 43.4 2.17 0.22). Standards were 1.2-diolein. 1.3-diolein. 1-oleoylglycerol, 2-oleoylglycerol. arachidonic acid. linoleic acid. phosphatidylinositol. and phosphatidylcholine. Those standards were run on the same TLC plate. The average ratio of sample migration to solvent front (R,) for 1.2-diolein was 0.59. No other standard had a similar R, (mono-oleoylglycerol R, = 0.32). The X-ray film was exposed to the TLC plates. the radioactive spots were scraped. Filtercount solubilizer and scintillation solution (Packard Instrument Co.. Downers Grove. Illinois) were added. and radioactivity was determined in a Packard Minaxi scintillation counter. The results were reported as disintegrations per min (dpm) at the various stages of exflagellation.

RESULTS

Gametocytes cultured in the presence of [¹H]myoinositol were transferred to a suspended animation medium (Carter and Nijout. 1977). which suppressed emergence from the erythrocytes and exflagellation. Exflagellation of the microgametocytes was initiated by removing them from this medium by centrifugation and resuspending them in complete medium containing 25 mEq sodium bicarbonate (Carter and Nijout. 1977). Thereafter. development of flagellae was monitored under 400× phase contrast microscopy. Figure 1A shows the kinetics of flagellar development in a typical experiment. The first exflagellations usually occurred 7–12 min after resuspension. The point of maximum exflagellation ranged from 14 to 17 min after resuspension: by 35 min there were no new exflagellations. As the process of exflagellation progressed. samples were taken for HPLC separation (Dean and Moyer. 1988) and quantification of radio-

FIGURE 1. Correlation of the kinetics of exflagellation with the production of inositol (Ins) (poly)phosphates. A. Exflagellation status after addition of bicarbonate was monitored by phase contrast microscopy (400×). Exflagellation grades were assigned according to the following traditional grading scheme: +1 (1–2/field). +2 (>4/field). +3 (>12/field). and +4 (>20/field. designated as maximal exflagellation). B. C. Four experiments were averaged to determine the appearance of the following inositol (poly)phosphates produced upon addition of bicarbonate: Ins(1,4,5)P₃ (triangles). Ins(4)P (squares). Ins(1,3,4,5)P₄ (solid circles). Ins(1)P (open circles). glycerophosphatidylinositol 4-phosphate (solid bar). The elution time (min) of standard inositides appeared as follows (see Fig. 2 for structures): Ins(1)P (27.1). Ins(4)P (32). Ins(1,3)P. (57). Ins(1,4)P. (60.6). Ins(2,4)P. (64.8). Ins(1,3,4)P₃ (82). Ins(1,4,5)P₃ (88). Ins(1,3,4,5)P₄ (102.3). GPI (13.4). GPIP (49). GPIP. (77.4).

PATHWAY A **PATHWAY B**

INS (1,4,5) P3

INS (1,3,4,5) P4

INS (1,4) P2

INS (1,3,4) P3

INS (4)P

INS (1,3) P2

INS (1) P

INOSITOL
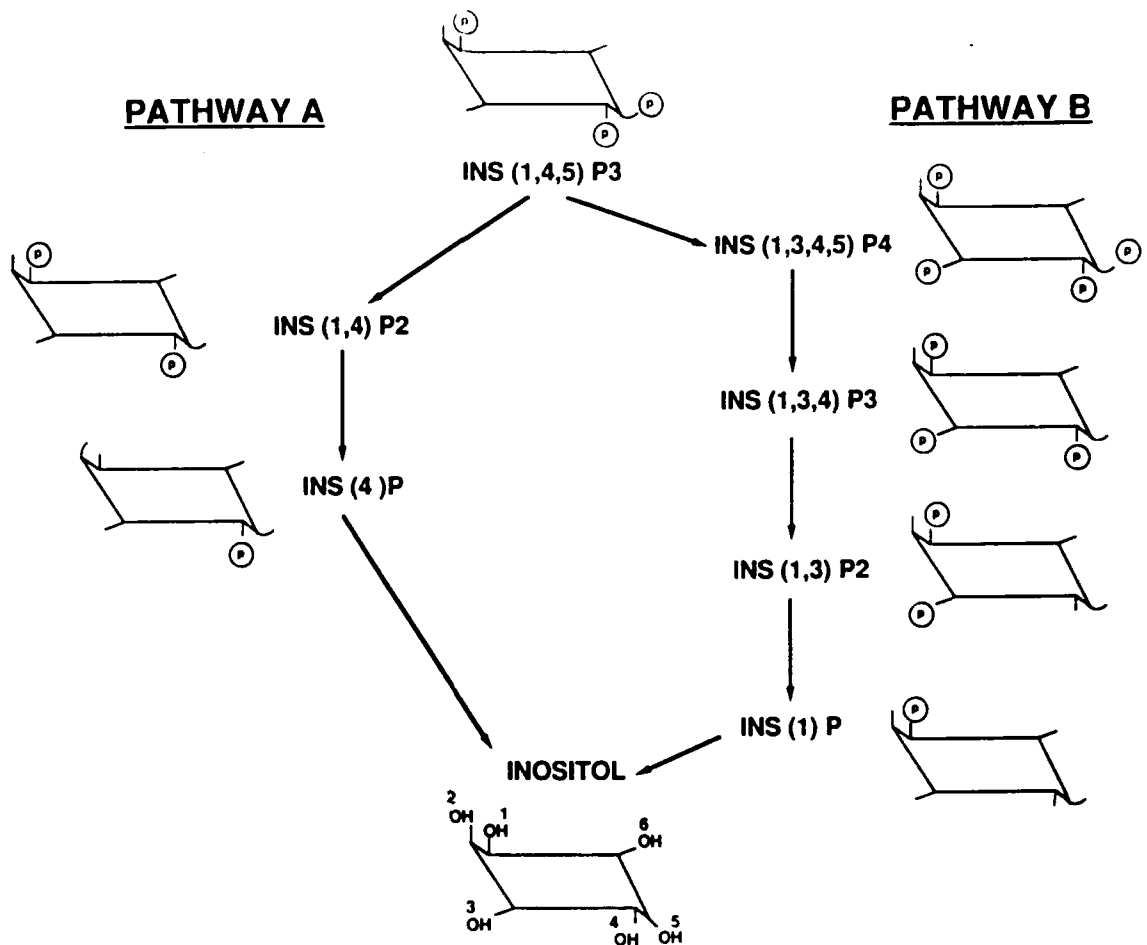
FIGURE 2. Schematic representation of possible degradative pathways of Ins(1,4,5)P$_3$. Pathway A shows the stepwise degradation by phosphatases of Ins(1,4,5)P$_3$. In pathway B, an energy-dependent pathway, specific kinases produce the tetrakisphosphate that is degraded eventually to Ins(1)P (Berridge, 1987, 1988; Shears, 1989).

activity associated with various inositol (poly)phosphates. The timing of sampling for biochemical analysis was based on the microscopic evaluation of microgamete development, except for the first few samples. The first sample, taken 30 sec after addition of bicarbonate ion buffer, showed that inositol (1,4,5)triphosphate (Ins[1,4,5]P$_3$) was just being formed, whereas at 60 sec its production had soared and remained high until exflagellation began (Fig. 1B). Samples taken at maximum exflagellation contained no detectable Ins(1,4,5)P$_3$.

On examination of the HPLC elution profiles, it became apparent that the immediate pathway activated was degradation to inositol (1,4)bis-phosphate (Ins[1,4]P$_2$) and subsequently inositol 4-phosphate (Ins[4]P) (pathway A, Fig. 2). Ins(4)P was not detected at 30 or 60 sec but had reached

its maximal detectable level when the first exflagellations appeared (Fig. 1B); minor amounts were detected at maximum exflagellation. In contrast, low levels of inositol (1,3,4,5)tetrakis-phosphate (Ins[1,3,4,5]P$_4$) (pathway B, Fig. 2) were detected upon appearance of the first exflagellations (Fig. 1B). Its production rose slightly at 17 min and reached its maximum at 30 min when exflagellation had virtually ceased (around 30 min). The major metabolite produced, inositol 1-phosphate (Ins[1]P), was detected in low levels at 0 time and its production closely paralleled exflagellation (Fig. 1C). One class of metabolite, glycerophosphoinositol (GPI), the 4-PO$_4$ (GPIP), and the (4,5)bis PO$_4$ (GPIP$_2$), was detected only at the period of maximal exflagellation (Fig. 1C).
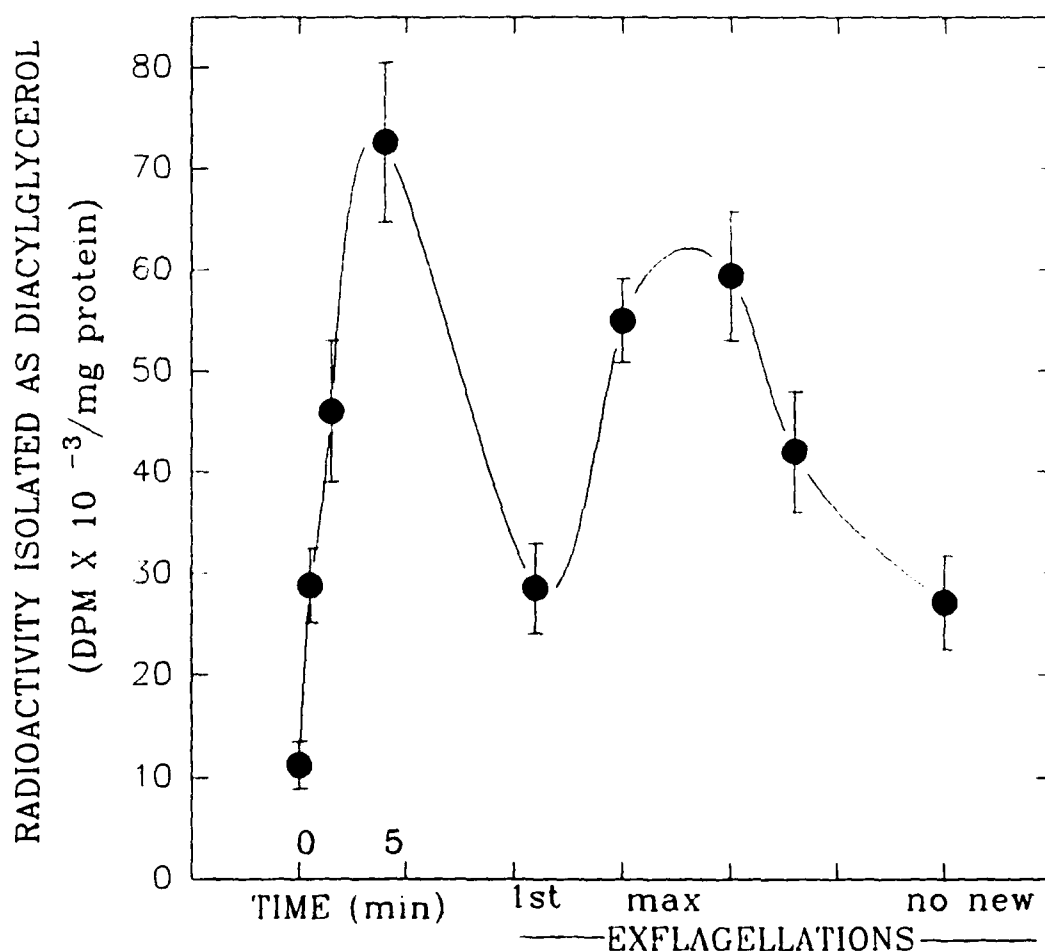
Experiments to determine the products of ino-

FIGURE 3. Production of diacylglycerol during exflagellation of microgametocytes. Cultures of gametocytes were prepared as described in the Materials and Methods section. During the maturation step, [³H]glycerol was added instead of radioactive inositol. Timed samples were taken as described in the Materials and Methods section, and the radioactivity that coeluted (TLC) with diacylglycerol was determined. The results were reported as disintegrations per min (dpm) at the various stages of exflagellation (defined in the legend to Fig. 1A).

sitol (poly)phosphates by exflagellating gametocytes were done 4 times. The same pattern of metabolite production was observed in each. Because of differences in the timing of maximal exflagellation (ranging from 12 to 18 min), the data are averaged to show inositol metabolite production at the various exflagellation stages; exflagellation stage, not time determined when samples were taken for analysis. Control experiments included early stage V *P. falciparum* gametocytes that did not exflagellate and produced no inositol (poly)phosphates when subjected to bicarbonate buffers.

To determine the kinetics of diacylglycerol (DAG) production, gametocytes were cultured as described previously except that [³H]glycerol was added during the gametocyte maturation step. As for inositol phosphate determinations, sam-

ples taken were based on status of exflagellation. Figure 3 shows the radioactivity that cochromatographed (by TLC) with DAG. There were 2 waves of DAG production, the first of which corresponded with the accumulation of $Ins(1,4,5)P_3$ (Fig. 1B) and the second to the accumulation of $Ins(1)P$ (Fig. 1C). At $T = 0$, both $Ins(1)P$ and DAG were present at low levels and were the only metabolites measured that were found to be generated prior to stimulation.

DISCUSSION

The first event in exflagellation is a rounding up of the crescent-shaped gametocytes and the emergence of the parasite from the erythrocyte. Even though the formation of $Ins(1,4,5)P_3$ preceded exflagellation, high levels of this com-

pound persisted into the time of microgamete release. Hence, Ins(1,4,5)P, may not only initiate the early events of rounding and emergence but also may play a role in the later nuclear events.

Exflagellation normally occurs between 7 and 20 min after exposure to ambient air, probably due to the nonsynchrony of the gametocytes in terms of maturity. This may explain the rather long period over which Ins(1,4,5)P, was detected in these cultures (7 min) in contrast to 3 min for other human cell culture systems (Berridge, 1987; Dean and Moyer, 1988; Catt and Balla, 1989; Changya et al., 1989). The formation of Ins(1,4,5)P, has been shown in many systems to be responsible for calcium mobilization (Berridge, 1988; Joseph and Williamson, 1989). Previous experiments with caffeine which elevates Ca²⁺ levels, showed that xanthine oxidase inhibitors can bypass the obligate requirement for bicarbonate and trigger exflagellation of microgametocytes of *Plasmodium gallinaceum*, an avian parasite (Martin et al., 1978).

Also investigated was the enzymatic pathway by which Ins(1,4,5)P, was degraded by the microgametocyte. Two possibilities have been shown to exist (Shears, 1989) as summarized in Figure 2. Using pathway A, Ins(1,4,5)P, is degraded by specific phosphatases to produce Ins(1,4)P₂; subsequently Ins(4)P. Pathway B, an energy-dependent route, results in the formation of Ins(1,3,4,5)P,. Other tetrakisphosphates have been identified in other systems (Shears, 1989); however, Ins(1,3,4,5)P, was the only tetrakisphosphate detected in the gametocyte cultures. This tetrakisphosphate is degraded to Ins(1,3,4)P, and then to Ins(1)P. On examination of the HPLC elution profiles, it became apparent that degradation of Ins(1,4,5)P, primarily proceeded via pathway A (appearance of Ins[4]P, Fig. 1B) until exflagellation approached its maximum, but then switched to pathway B (characterized by Ins[1,3,4,5]P, production, Fig. 1B), during maximal exflagellation and through the remainder of the experiment. These observations suggest a role for calcium mobilization extending beyond the initial stimulation (which resulted in the immediate production of Ins[1,4,5]P,) since Ins(1,3,4,5)P₄ is thought to act in synergy with Ins(1,4,5)P, by transferring calcium from an Ins(1,4,5)P,-insensitive pool to a sensitive pool (Berridge and Irvine, 1989; Changya et al., 1989) and to prolong calcium mobilization by increasing the lifespan of Ins(1,4,5)P, (Shears, 1989). The most abundant metabolite, Ins(1)P, does not

make any contribution to calcium mobilization, and its appearance may be more important as a reflection of DAG production. It can arise from a number of parent compounds in addition to Ins(1,3,4,5)P,. One such example is its release by phospholipase C from phosphatidylinositol, rather than its polyphosphates.

Studies of phospholipase C specificity toward the (poly)phosphoinositides have shown that at high calcium concentrations, enzymatic preference shifts from phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) to phosphatidylinositol 4-phosphate and finally to phosphatidylinositol (PtdIns) (Wilson et al., 1984). The phospholipase C-generated products from each substrate are Ins(1,4,5)P,, Ins(1,4)P₂; and Ins(1)P, respectively. Only 1 of those products, Ins(1,4,5)P,, stimulates calcium mobilization, and its production was diminishing by the time exflagellations began (ca. 7 min). Ins(1)P, a metabolite maximally produced between 7 and 35 min, suggests that the enzyme specificity had shifted from PtdInsP₂ to PtdIns. Although DAG was a coproduct with both Ins(1,4,5)P, and Ins(1)P (Nishizuka, 1984), there was ca. 10 times more production of the inactive Ins(1)P than of calcium mobilizing Ins(1,4,5)P,. Since DAG is a potent activator of protein kinase C (PKC), this may mean that phosphorylation plays a predominant role during the phase of maximal exflagellation. Interestingly, Ins(1)P and DAG production almost exactly paralleled the rise in exflagellation.

The first peak of DAG production could be attributed to a combination of its baseline release from phosphatidylinositol (along with Ins(1)P) as well as the stimulated release (concomitantly with Ins[1,4,5]P,) from phosphatidylinositol 4,5-bisphosphate. The second peak of DAG production, correlated with the production of Ins(1)P.

The other major inositol phosphate metabolite detected was GPIP₂. Unlike the other inositol (poly)phosphate metabolites, which are initially released from phosphoinositides by phospholipase C, this product results from the action of phospholipases A₂ and A₁, which remove both fatty acids from the phospholipid leaving the glycerol backbone with Sn-3 phosphodiester linkage to inositol (or its polyphosphates). In 3 experiments this product (and to a lesser extent, glycerophosphatidylinositol 4-phosphate) was detected only at the peak of exflagellation. This compound is suspected of mobilizing calcium from intracellular stores, although Ogawa and Harafuji (1989), using sarcoplasmic reticulum,

showed it to be incapable of releasing intracellular calcium in that system. In addition to the formation of the GPIPs, free fatty acids would be released. The Sn-2 fatty acid is frequently arachidonic acid, and its metabolites are known to have far-reaching effects on biological systems. Arachidonate metabolites may well be as important as the other part of the molecule, the GPIPs, whose role is poorly defined. The biological function of these signals cannot be discerned from these experiments as no attempt was made to link them to specific functions.

The similarly of the kinetics of phosphoinositide hydrolysis products in responsive plasmodial and mammalian cells suggests that the biochemical pathways for signal transduction may be highly conserved. Therefore, the more specialized *P. falciparum* parasite could well serve as a model for investigating these important events.

ACKNOWLEDGMENTS

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